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Carbohydrate Research 327 (2000) 463–481

CARBOHYDRATE RESEARCH

End-group determination of oligosaccharides: a gas chromatography–mass spectrometry/mass spectrometry method for distinguishing between all D-aldohexoses and D-ketohexoses☆,☆☆

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Received 5 October 1999; accepted 10 February 2000

Abstract

A method for end-group determination of oligosaccharides is described, which involves conversion of the reducing monosaccharide into a 1-deoxy-1-hydrazinohexitol heptaacetate (aldohexoses) or an epimeric pair of 2-deoxy-2-hydrazinohexitol heptaacetates (2-ketohexoses). Products are linear and unique to each aldohexose or ketohexose. Methods are reported for separation of all stereoisomers of the derivatives on single columns by gas chromatography. Gas chromatography–mass spectrometry/mass spectrometry with electron-impact ionization enabled the 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates to be independently identified in each others presence. Chemical-ionization mass spectrometry/mass spectrometry permitted derivatives to be identified in subpicomolar quantities. The non-acetylated compounds could also be identified as their hydrochlorides by ^{1}H NMR spectroscopy.

Keywords: Carbohydrate; Oligosaccharide; End-group determination; Gas chromatography–mass spectrometry; Monosaccharide analysis

1. Introduction

In the development of a chemistry enabling monosaccharides to be removed sequentially from the reducing end of oligosaccharides [2–4], a procedure to sensitively identify the stereochemistry of the monomer at the reducing end was necessary. A derivative was sought having the following preferred properties. First, the derivative should uniquely define each original reducing-end sugar. This is not possible through reduction of the reducing monosaccharide to its alditol; several sugar sets may give rise to the same alditol. For instance, D-glucose, L-gulose, D-fructose and L-sorbose all reduce to yield (at least some) D-glucitol. Second, it would be preferred that the derivative be linear, rather than having multiple cyclic forms. Third, the derivative should survive the conditions of hydrolysis–solvolysis used to cleave glycosidic linkages. Fourth, the derivative should be small enough to permit complete physical separation based solely on the stereochemistry of the

☆ Part IV, Sequential removal of monosaccharides from the reducing end of oligosaccharides.

☆☆ A preliminary report has been presented [1].

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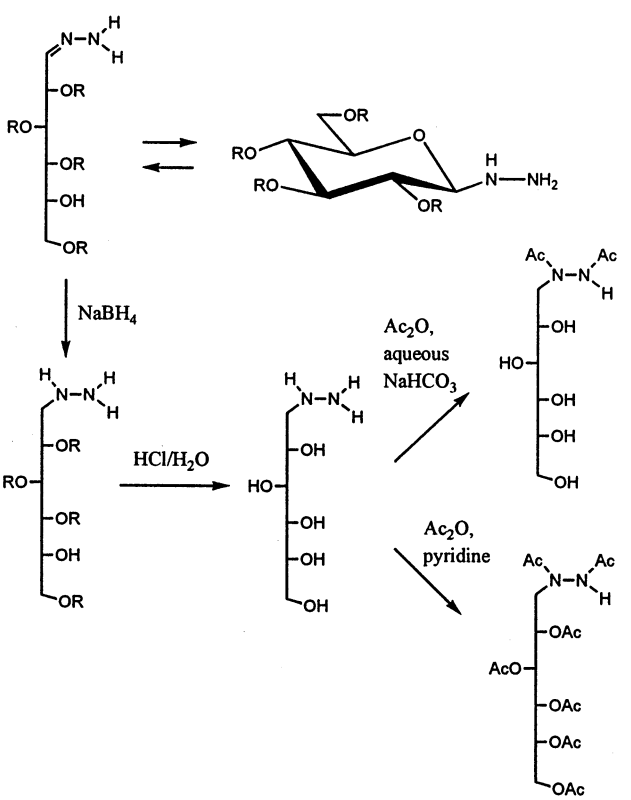
0008-6215/00/$ - see front matter
PII: S0008-6215(00)00058-6

monomers. Fifth, the derivatization should be near-quantitative and straightforward. Sixth, standards should be stable. Seventh, it would be preferred that the derivative permit regioisomers (ketoses and aldoses) to be readily identified in each others presence. Finally, for quantitative purposes, the derivatization protocol should be amenable to inclusion of an internal standard having an isotopic mass difference. Here, we demonstrate that 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates meet the foregoing needs for oligosaccharide end group determination when combined with gas chromatography–mass spectrometry/mass spectrometry.

2. Results and discussion

The reactions for preparation of 1-deoxy-1-hydrazinohexitols, 2-deoxy-2-hydrazinohexitols, and their *N*,*N*-diacetyl and heptaacetyl derivatives are illustrated in Scheme 1 for the 1-deoxy-1-hydrazino derivatives. Monosaccharides and oligosaccharides having a reducing end were treated at room temperature with anhydrous hydrazine in great excess to avoid azine formation. Upon removal of excess hydrazine and immediately dissolving the product in D_2O (unbuffered), all sugars were found by NMR spectroscopy to be converted, essentially quantitatively, into hydrazones and their tautomeric cyclic glycosylhydrazines (Tables 1 and 2). Over a period of a few hours, the ratios of acyclic and cyclic forms changed according to the individual sugar, and traces of the free reducing sugar appeared, indicating a slight hydrolysis in water. However, the sugar hydrazones/cyclic glycosylhydrazines predominated (97–98%) at equilibrium, even after 72 h. This equilibrium is dependent to some extent on additional hydrazine in the syrup upon dissolution, but clearly strongly favors hydrazone/cyclic glycosylhydrazine formation in unbuffered D_2O solution (*p*D 83–9.0).



Scheme 1. Derivatization chemistry for end-group determination of oligosaccharides. R represents H or a glycosidic substituent. Stereochemistry along the backbone may be variable (not shown). Only the reactions with aldohexoses are shown.

Other studies of the formation and stability in aqueous solution of some of the sugar hydrazones/glycosylhydrazines have been reported [3,5–8], and are in agreement with our observations that these molecules are quite stable in unbuffered aqueous solution. Decreasing the pH to 6 [5,7] leads to a much greater preponderance of cyclic forms as well as slow hydrolysis to free sugars. Reported here for all aldohexose hydrazones, the H-1 signals of the major acyclic form in D_2O were downfield (δ 7.2–7.4 ppm, Table 1) and were attributed to the *E* isomer based on previous correlations for *E* and *Z* forms of sugar oximes [9]. For all aldohexose hydrazones, a second weak downfield doublet was also observed (δ 6.6–6.8 ppm), which was attributed to the Z isomer. It should be pointed out that these assignments, for oximes, were based primarily on shielding/deshielding effects that were expected to contribute to the differences in chemical shifts, and that hydrazone isomers have been assigned by analogy [5,7]; to our knowledge, heteronuclear $^3J_{H,N}$ or $J_{C,N}$ constants for aldose hydrazone isomers have not been measured. The predominant cyclic forms were β-pyranosyl (Table 1), having H-1 doublets-

Table 1
^{1}H NMR data of aldohexose hydrazones/cyclic glycosylhydrazines [a]

Sugar	Chemical shift (δ, ppm)							Coupling constant (Hz)							Conformation [b]
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6b,6b}$	
D-All	7.323	4.391	3.883	3.584	3.893	3.794	3.653	6.4	4.6	7.1	5.6	3.2	7.2	−11.9	acyclic
D-All	4.266	3.459	4.147	3.583	3.70 [e]	3.888	3.69 [c]	9.3	3.1	3.0	9.8	[d]	[d]	[d]	β-pyr, $^4C_1$
D-Alt	7.333	4.418	3.758	3.808	3.910	3.793	3.668	5.4	2.8	8.1	[d]	3.2	7.4	−11.9	acyclic
D-Alt	4.432	3.797	4.007	3.762	[d]	[d]	[d]	≤1.0	3.3	3.5	[d]	[d]	[d]	[d]	β-pyr, $^4C_1$
D-Gal	7.366	4.440	3.761	3.684	3.961	3.678	3.678	5.3	2,4	9.2	1.5	[c]	[e]	[e]	acyclic
D-Gal	3.988	3.486	3.651	3.928	3.68 [f]	3.778	3.728	9.0	9.7	3.5	≤1.0	8.0	4.2	−11.7	β-pyr, $^4C_1$
D-Glc	7.245	4.285	3.907	3.542	3.752	3.818	3.622	6.4	7.5	1.7	8.6	2.9	6.3	−11.9	acyclic
D-Glc	4.035	3.260	3.491	3.367	3.417	3.906	3.718	9.0	9.2	8.8	9.8	2.2	5.6	−12.2	β-pyr, $^4C_1$
D-Gul	7.313	4.219	3.789	3.779	3.822	3.714	3.619	6.5	6.6	[g]	4.9	4.0	6.6	−11.8	acyclic
D-Gul	4.283	3.659	4.033	3.809	3.933	3.757	3.716	9.4	3.4	3.6	≤1.0	7.7	4.7	−12.2	β-pyr, $^4C_1$
D-Ido	7.289	4.316	3.791	3.676	3.834	3.710	3.629	5.9	5.6	4.2	4.4	4.2	6.9	−11.7	acyclic
D-Man	7.341	4.191	3.899	3.76 [h]	3.76 [h]	3.849	3.661	6.4	8.1	≤1.0	[h]	[h]	[h]	−11.8	acyclic
D-Man	4.235	3.914	3.631	3.543	3.355	3.936	3.724	≤1.0	3.4	9.7	9.7	2.3	6.6	−12.2	β-pyr, $^4C_1$
D-Tal	7.325	4.423	3.891	3.539	3.934	3.649	3.649	6.4	3.7	9.0	1.7	[c]	c	[e]	acyclic
D-Tal	4.178	3.862	3.752	3.88 [f]	3.603	3.857	3.539	≤1.0	3.2	3.4	≤1.0	8.0	4.2	−11.8	β-pyr, $^4C_1$

[a] For a solution in D_2O at 27 °C relative to internal DSS at 0.000 ppm. Data was derived from 1-D and 2-D (gCOSY) experiments.
[b] Two major tautomers were observed for most products, pyranosyl denoted pyr.
[c] Could not be determined to higher resolution due to partial strong coupling of H-5 and H-6b.
[d] Not determined due to severe multiplet overlap and/or partial strong coupling.
[e] H-6a and H-6b were strongly coupled; 1/2 ($J_{5,6a}+J_{5,6b}$) = 6.4 Hz.
[f] Could not be determined to higher resolution due to severe multiplet overlap.
[g] Could not be determined, or not determined to higher resolution due to partial strong coupling of H-3 and H-4.
[h] Partial strong coupling of H-4 and H-5, with virtual coupling effects on H-3, H-6a and H-6b.

Table 2
^{1}H NMR data of 2-ketohexose hydrazones/cyclic glycosylhydrazines [a]

Sugar	Chemical shift (δ, ppm)							Coupling constant (Hz)						Conformation [b]
	H-1a	H-1b	H-3	H-4	H-5	H-6a	H-6b	$J_{1a,1b}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$	
D-Fru	4.223 [c]	4.223 [c]	4.943	3.815	[d]	[d]	[d]	[c]	2.0	[d]	[d]	[d]	[d]	acyclic
D-Fru	4.389	4.320	4.498	3.738	[d]	[d]	[d]	−14.3	2.8	[d]	[d]	[d]	[d]	[e]
D-Psi	4.291	4.192	4.904	3.958	3.75 [f]	[g]	[g]	−13.9	6.7	6.4	[g]	[g]	[g]	acyclic
D-Psi	4.386	4.348	4.371	3.818	[g]	[g]	[g]	−14.1	6.3	6.4	[g]	[g]	[g]	[e]
D-Sor	4.279	4.168	4.804	3.945	3.639	[h]	[h]	−14.0	6.9	2.3	[h]	[h]	[h]	acyclic
D-Sor	4.384	4.328	4.380	3.787	3.691	[h]	[h]	−14.3	6.1	3.4	[h]	[h]	[h]	[e]
D-Tag	4.310	4.216	4.857	3.879	3.972	3.680 [i]	3.680 [i]	−14.1	9.6	1.4	[i]	[i]	[i]	acyclic
D-Tag	4.373 [c]	4.373 [c]	4.282	3.733	3.905	3.68 [f]	3.67 [f]	[c]	8.5	2.2	7.1	5.4	[f]	[e]

[a] For a solution in D_2O at 27 °C relative to internal DSS at 0.000 ppm. Data was derived from 1-D and 2-D (gCOSY) experiments.
[b] Two major tautomers were observed for each product; acyclic tautomers were assigned based on the downfield position of H-3.
[c] Strongly coupled.
[d] Not determined due to partial strong coupling of both H-4, H-5 pairs, and severe spectral overlap of H-5, H-6a and H-6b signals between δ 3.6 and 3.8 ppm.
[e] The second tautomer was cyclic, and probably furanosyl in all cases, but anomericity could not be determined due to lack of an anomeric proton.
[f] Values were assigned only to two decimal places due to partial strong coupling and/or severe spectral overlap.
[g] Not determined due to partial strong coupling and severe spectral overlap of all H-5, H-6a and H-6b signals between δ 3.6 and 3.7 ppm.
[h] Not determined due to partial strong coupling and severe spectral overlap of all H-5, H-6a and H-6b signals between δ 3.6 and 3.8 ppm.
[i] Strongly coupled H-6a and H-6b; $1/2\ (J_{5,6a}+J_{5,6b}) = 6.4$ Hz.

between 3.9 and 4.5 ppm, except for idose, which was almost entirely the open-chain tautomer. For all ketohexoses, hydrazone products were generated in essentially quantitative yields, as evaluated by ^{1}H NMR immediately after dissolving in unbuffered D_2O.

Two major tautomers were also observed, an open-chain and cyclic form (Table 2). Due to the lack of an anomeric proton on ketoses, the open-chain form was assigned based on the downfield position of the H-3 signal relative to that of the cyclic form, similar to the downfield shift of H-2 signals of the open-chain forms of the aldose hydrazones as compared with their pyranosyl tautomers (Table 1). Also, owing to the lack of an anomeric proton, it was not possible to determine, without further experiments, whether cyclic forms were α or β. From the *J* couplings that could be determined, cyclic furanose forms were indicated, although for the fructose product, evidence for this was weak because only the $J_{3,4}$ coupling was measurable.

Reduction of sugar hydrazones was achieved using high concentrations of $NaBH_4$ (10%, w/w) in water. The ensuing products, for the aldohexoses, were 1-deoxy-1-hydrazinoalditols and for the ketohexoses were a 2-epineric pair of 2-deoxy-2-hydrazinoalditols. For all monosaccharides and oligosaccharides, a small quantity (2–4%) of the alditol or 2-epimeric pair of alditols (ketoses) was also present, indicating that hydrolysis occurred to a small extent during the reduction. Lowering the borohydride concentration gave more of the alditol product, so the reaction yields depend on relative rates of reduction of the sugar hydrazones and the reducing sugars themselves, and the rate of conversion of the hydrazones to the free sugars. For practical purposes, a 10% solution of sodium borohydride was sufficient for $>96\%$ reaction yields (as monitored by GC–MS of the peracetylated products, below).

Following acidification of the reaction mixture with acetic acid, a high concentration of boric acid and sodium acetate precluded simple isolation of the 1-deoxy-1-hydrazinohexitols. It was found that *N*,*N*-diacetylation could be performed directly on the aqueous product by adding $NaHCO_3$ and Ac_2O, which permitted sodium to be removed on Dowex AG50W (H^+ form) columns. Yields of 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)hexitols using the described protocol were essentially quantitative, but yields of 2-deoxy-(2-*N*,*N*-diacetylhydrazino)hexitols were low (10–15%). The inner nitrogen of 2-deoxy-2-hydrazinohexitols was less amenable to acetylation under aqueous conditions, and after acetylation of the terminal nitrogen, the inner nitrogen, possibly due to steric crowding, was not fully acetylated. The singly acetylated molecules bound, almost totally, to Dowex AG5OW (H^+ form) columns. The *N*,*N*-diacetylhydrazino derivatives chromatographed (HPLC) as single peaks, but were not suitable for NMR analysis. ^{1}H NMR of a few of them showed broad signals, indicative of molecular interconversions on the same time frame (1–2 s) as the accumulation of free-induction decays. Mass spectra (FAB) gave an $(M+H)^+$ ion at m/z 281, and, in the presence of sodium ion, a sodium adduct $(M+Na)^+$ at m/z 303. High-performance liquid chromatography (HPLC) purified *N*,*N*-diacetylhydrazino derivatives were readily converted into the heptaacetates by O-acetylation in pyridine–Ac_2O (see Section 4), but the heptaacetates were more easily prepared directly after reduction of the sugar hydrazones, as described below. Further details concerning the *N*,*N*-diacetylhydrazinohexitols are restricted to Section 4.

Alternatively, boric acid could be removed in the presence of sodium acetate as trimethylborate by repeated evaporations from methanol–acetic acid and methanol. The large excess of sodium acetate remaining catalyzed peracetylation upon addition of Ac_2O, a commonly used combination for peracetylation of partially methylated alditols during the permethylation analysis of oligosaccharides [10,11]. The resultant products, in nearly quantitative yields, were the 1-deoxy-1-hydrazinohexitol heptaacetates (from aldoses) and the epimeric pairs of 2-deoxy-2-hydrazinohexitol heptaacetates (from ketoses), which were readily separated from sodium acetate by partitioning between chloroform–water. Other contaminating products at this stage were small quantities (2–4%) of the respective alditol acetates, and *N*,*N*-diacetylhydrazine.

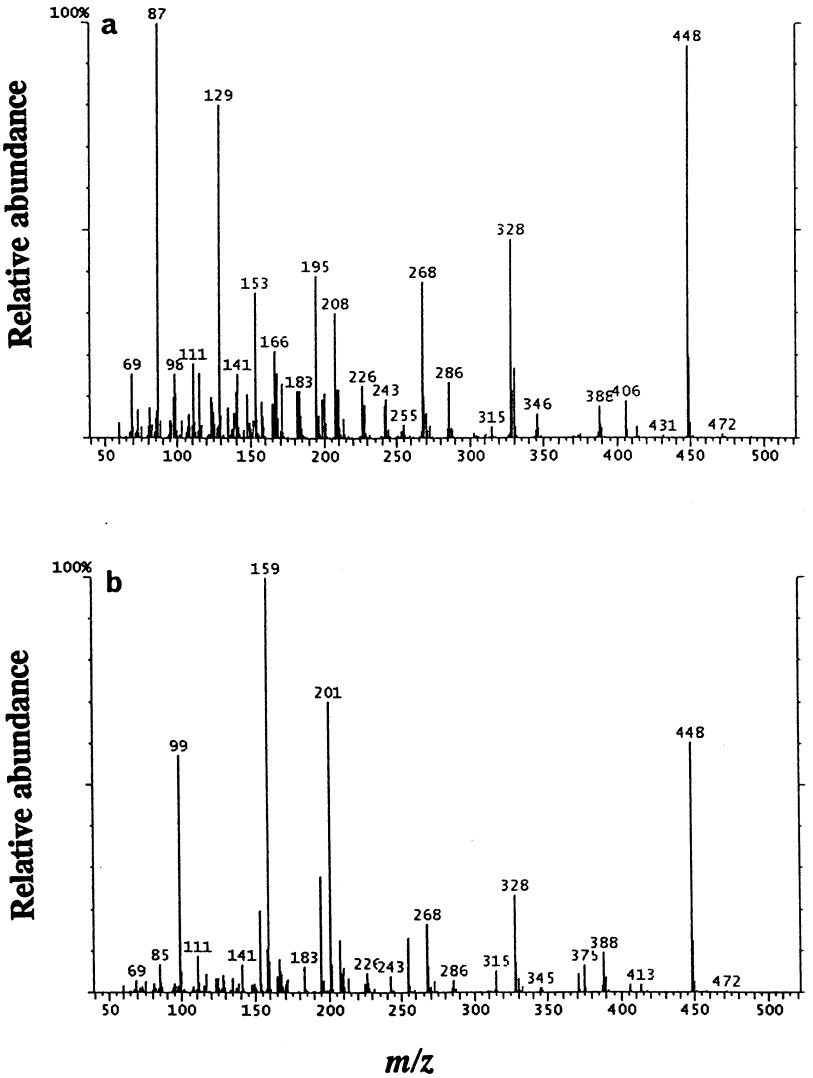
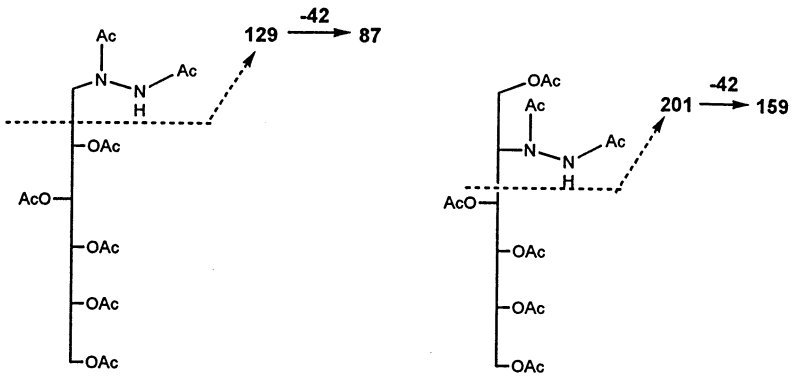


Fig. 1. Electron-impact mass spectra for 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates. Data were acquired in full-scan mode with the source at 225 °C; electron energy, 70 eV; trap offset, −10 V; automatic gain control target, 50; high mass adjust parameter, 65%. (a) The EI mass spectrum for a 1-deoxy-1-hydrazinohexitol heptaacetate. (b) The EI mass spectrum for a 2-deoxy-2-hydrazinohexitol heptaacetate. For each set of derivatives, spectra for all stereochemical variants were similar.

The heptaacetates were amenable to GC–MS and GC–MS/MS analyses. Fig. 1(a) and (b) shows the standard electron impact (EI) mass spectra of 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates obtained at 70 eV. Diagnostic backbone fragmentation sites are shown in Scheme 2. A remarkable feature of the fragmentation patterns is that the preponderant site of cleavage along the backbone of these molecules is between C-1 and C-2 for the 1-deoxy-1-hydrazinohexitol heptaacetates, and between C-2 and C-3 for the 2-deoxy-2-hydrazinohexitol heptaacetates, which just happen to be the sites needed to distinguish between them. This resulted in diagnostic abundant ions at m/z 129 and 87 for the 1-deoxy-1-hydrazinohexitol heptaacetates, and at m/z 201, 159 and 99 for the 2-deoxy-2-hydrazinoalditols. A careful examination of other expected masses for ions resulting from fragmentation at other backbone positions (m/z 417, 361, 345, 289, 273, 217, and 145) showed only traces of such ions, if observed at all. Fragmentation probably occurs between C-5 and C-6 to some extent (and/or between C-1 and C-2 for the 2-deoxy-2-hydrazinoalditol heptaacetates) because an ion series starting from 375 ($375 \rightarrow 315 \rightarrow 255 \rightarrow 195$) was observed, representing an initial loss of 73 +

42 (loss of a $-CH_2OAc$ and a ketene, followed by losses of acetates (-60). Another series of ions (*m*/*z* 448, 406, 388, 346, 328, 286, 268, 226, 208, 166) were generated by stripping of acetates and ketenes (-42) from the parent molecules (mass of 490). These ions were not diagnostic for regioisomers (aldohexoses vs. ketohexoses), although their ratios differed.

Electron-impact GC–MS/MS spectra of 1-deoxy-1-hydrazinohexitol heptaacetates were acquired for a number of selected ions; fragmentation of the diagnostic precursor ion at *m*/*z* 129 resulted in a loss of ketene (-42) to give a unique product ion at *m*/*z* 87. Similarly, selection of the key precursor ion at *m*/*z* 201 for the 2-deoxy-2-hydrazinohexitol heptaacetates gave, upon fragmentation, an abundant ion at *m*/*z* 159 due to loss of ketene, a minor ion at 141 due to loss of acetate, and a minor ion at 99 due to loss of both. Two major advantages to MS/MS analysis in real chro-



Scheme 2. Diagnostic backbone cleavages for the 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates observed during EIMS and EIMS/MS.

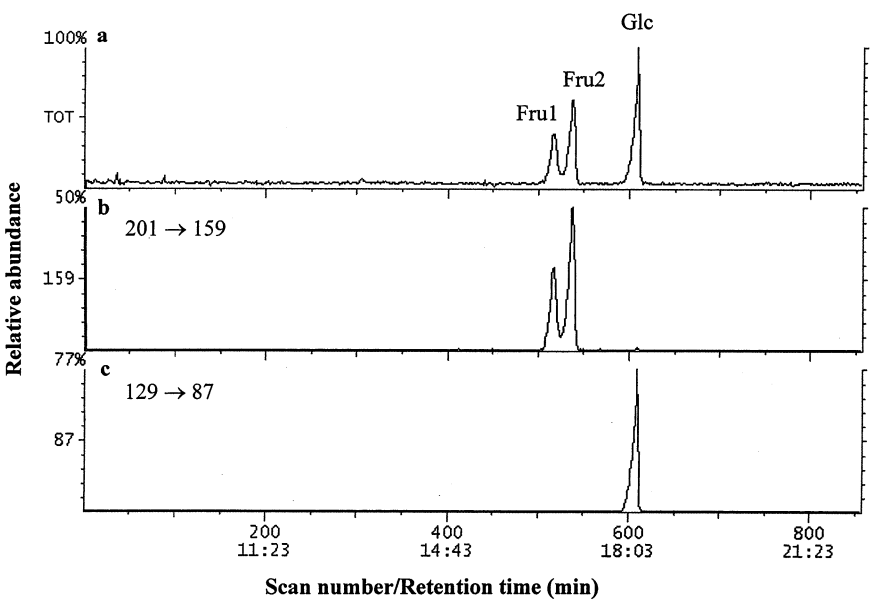


Fig. 2. Simultaneous GC–EIMS/MS analyses of 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates. Products from D-glucose and D-fructose were chromatographed on a 20 m DB-1701 column (0. 18 mm i.d., 0.4 μm film thickness) at a constant linear velocity of He of 30 cm/s. The injector temperature was 300 °C. The oven program was 90 °C for 1.5 min during transfer from the liner, then increased at 400/min to an isothermal plateau of 280 °C. Dual MS/MS was performed in the positive ion mode, with the automatic gain control target, 50; trap offset, -10 V; ion RF, 43. Precursor ions of *m*/*z* 129 and 201 were selected; isolation width, 2 amu; isolation time, 8 ms; resonance excitation RF voltage, 0.4 V for 15 ms; *q* value, 0.225. From the same chromatographic run are shown (a) the total ion current; (b) the product ion *m*/*z* 159 from the precursor ion *m*/*z* 201; (c) the product ion *m*/*z* 87 from the precursor ion *m*/*z* 129. The column was slightly overloaded to clearly demonstrate precursor-product ion specificity; slight 'fronting' of peaks resulted.

matographic time were, first, as shown in Fig. 2, that aldohexoses and ketohexoses can be readily distinguished from each other. Simultaneous selection of the diagnostic $129 \rightarrow 87$ and $201 \rightarrow 159$ conversions are highly characteristic for the 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates, respectively. Even if the molecules happen to comigrate, their presence can be ascertained. This permitted independent monitoring of aldohexose and ketohexose derivatives. Second, the practical limitation for detection of electron impact spectra, at least on the Thermoquest GCQ instrument, is the background level of ions from column bleed, which need to be subtracted from sample ions. Although columns may very well bleed ions of m/z 129 and 201 in small quantities, selection of the $129 \rightarrow 87$ and $201 \rightarrow 159$ conversions are highly unique to sample ions, which significantly increased sensitivity. Depending on the GC column and the ion chosen, increases in sensitivity ranged from about 5 to 20 fold, and were ultimately limited by noise of the detector and other inherent features of ion trap design and use, rather than column bleed.

Chemical ionization (CI) was also effective in generating ions from these derivatives. Three CI gases (methane, ammonia, and isobutane) were examined, but isobutane was the most effective for generating positive ions. Interestingly, isobutane was also effective in generating negative ions for mass analysis. Positive ion CI spectra using isobutane for 1-deoxy-1-hydrazinoalditol heptaacetates and 2-deoxy-2-hydrazinoalditol heptaacetates were similar, with an observable $(M + H)^+$ ion at m/z 491. The principal ion, however, was m/z 431, representing loss of an acetate, with much lesser quantities of other ions. CI was more sensitive than EI because fragmentation was minimal. Moreover, bleed of column ions tends to diminish significantly above about m/z 250, increasing the apparent detectability of the 431 ion relative to low mass ions. Selection of the m/z 431 ion for MS/MS yielded full spectra (Fig. 3(a) and (b)). For both the 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptacetates, essentially all product ions derived from the m/z 431 precursor resulted from stripping of acetates and ketenes, although ratios of the ions differed (spectra in Fig. 3(a) and (b) were from the same chromatographic run). These ratios, however, were similar among each class of compounds (that is, compounds derived from aldoses or ketoses). There were no observable cleavages along the backbone. Consequently, although CIMS/MS in the positive ion mode distinguishes the regioisomers when they appear as separate peaks, it is not possible to easily ascertain the presence of the 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates together should they happen to comigrate. Of the methods presented, positive ion CIMS/MS was the most sensitive for obtaining full spectra. In Fig. 4, the total ion current of a chromatographic separation of 300 femtomoles of each of six 1-deoxy-1-hydrazinohexitol heptaacetates is shown. Full spectra were similar to those in Fig. 3(a).

Negative-ion CI spectra using isobutane for 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates were also similar. Some of the $(M - H)^-$ ion, at m/z 489, was observed, but the most abundant ion found was m/z 447 $(M - H - 42)^-$, due to loss of ketene. Other ions of minor abundance were also observed, mainly representing additional losses of ketenes and/or acetates along the backbone. Selection of the m/z 447 ion for MS/MS gave full spectra (Fig. 5(a) and (b)). Negative-ion spectra using isobutane were not as sensitive as positive ion spectra, but were more sensitive than electron-impact spectra. The product ions resulting from the m/z 447 precursor represented losses due to stripping of ketenes and acetates from the backbone. Although there were consistent spectral differences in ion ratios between 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates, should they overlap chromatographically, it would be difficult to ascertain one in the presence of an abundance of the other.

Chromatography of the 1-deoxy-1-hydrazinohexitol heptaacetates on a 105-m DB-1701 column is shown in Fig. 6(a). All of the stereochemical variants of the D-aldohexose derivatives were separable. Useful shorter

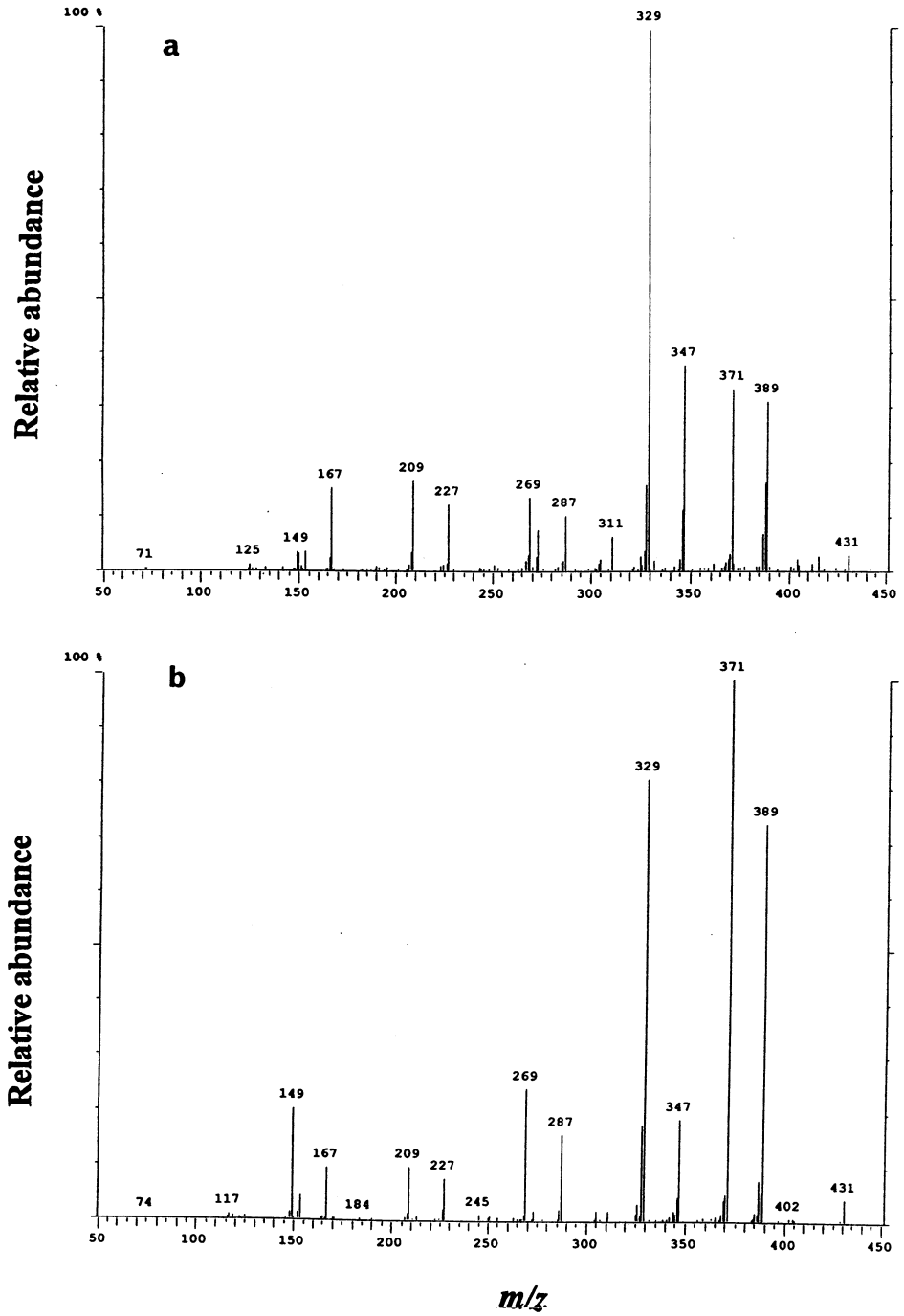


Fig. 3. Chemical ionization-MS/MS spectra in the positive ion mode for 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates, using isobutane as the CI gas, with selection of the major precursor ion m/z 431. (a) The CIMS/MS spectrum for a 1-deoxy-1-hydrazinohexitol heptaacetate. (b) The CIMS/MS spectrum for a 2-deoxy-2-hydrazinohexitol heptaacetate. For each set of derivatives, spectra for all stereochemical variants were similar. Spectra for (a) and (b) were from the same chromatographic run under identical conditions. Data were acquired in MS/MS mode; electron energy, 100 eV; source temperature 225 °C; iontrap offset, −7 V; automatic gain control target, 200; ion RF, 47. Precursor ions were selected with an isolation width, 2 amu; isolation time, 8 ms; resonance excitation RF voltage, 0.65 V for 30 ms; q value, 0.225. Product ions were scanned over the ranges shown.

columns, among a number that were examined, were DB-17 and DB-1301 (Fig. 6(b) and (c)), which separated six of the eight derivatives but with different patterns of overlap. They have utility for rapid separations of less complex mixtures. A 100-m DB1 column was found to separate all eight stereochemical variants of the 2-deoxy-2-hydrazino-D-hexitol

heptaacetates (Fig. 6(d)). Another useful column was DB1 (60 m, 0.25 mm i.d., 0.25 μm film thickness), which separated the more frequently encountered Glc, Man, and Gal from all other derivatives. However, three of the other more rare sugars, Alt, Gul and Ido, overlapped (data not shown). An additional shorter column for ketose derivatives was DB-17 (as above), which resolved the D-Psi, D-Fru, and D-Sor derivatives, but which showed overlap of the D-Tag derivatives with one D-Sor and one D-Fru derivative (data not shown).

NMR ($CDCl_3$) was not well suited for analysis of the heptaacetate derivatives. Signals were either broad, indicating interconversion on the NMR time scale, or two or three conformers were observed. The data indicate that the nitrogens are sp^2 hydridized, like amide nitrogens, and that carbonyl carbons and hydrazide nitrogens probably exist in planar variants with substituents cis or trans to each other, giving rise to relatively stable conformers at room temperature. Clearly, at temperatures at which the molecules migrate on GC columns, these interconversions were so rapid that single peaks were observed.

1-Deoxy-1-(*N*,*N*′-diacetylhydrazino)hexitols, 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)hexitols, 1-deoxy-1-hydrazinohexitol heptaacetates, and 2-deoxy-2-hydrazinohexitol heptaacetates were all deacetylated upon treatment with 2.0 M HCl for 3.5 h at 100 °C. The products, 1-deoxy-1-hydrazinohexitol hydrochlorides or 2-deoxy-2-hydrazinohexitol hydrochlorides, were isolated by binding of the protonated products to columns of Dowex-AG50W, H^+ form, followed by elution with 4.0 M HCl. For the 1-deoxy-1-hydrazinohexitol hydrochlorides, ^1H NMR gave well-resolved spectra of single species (Table 3). Gradient COSY spectra enabled complete correlations to be made. Assignments for the 2-deoxy-2-hydrazinohexitol hydrochlorides were made similarly (Table 4). The specific stereochemistry at C-2 attributed to each set of assignments was not possible without further studies. Electrospray mass spectrometry yielded the $(M + H)^+$ ions, m/z 197, for every 1-deoxy-1-hydrazinohexitol and 2-deoxy-2-hydrazinohexitol hydrochloride.

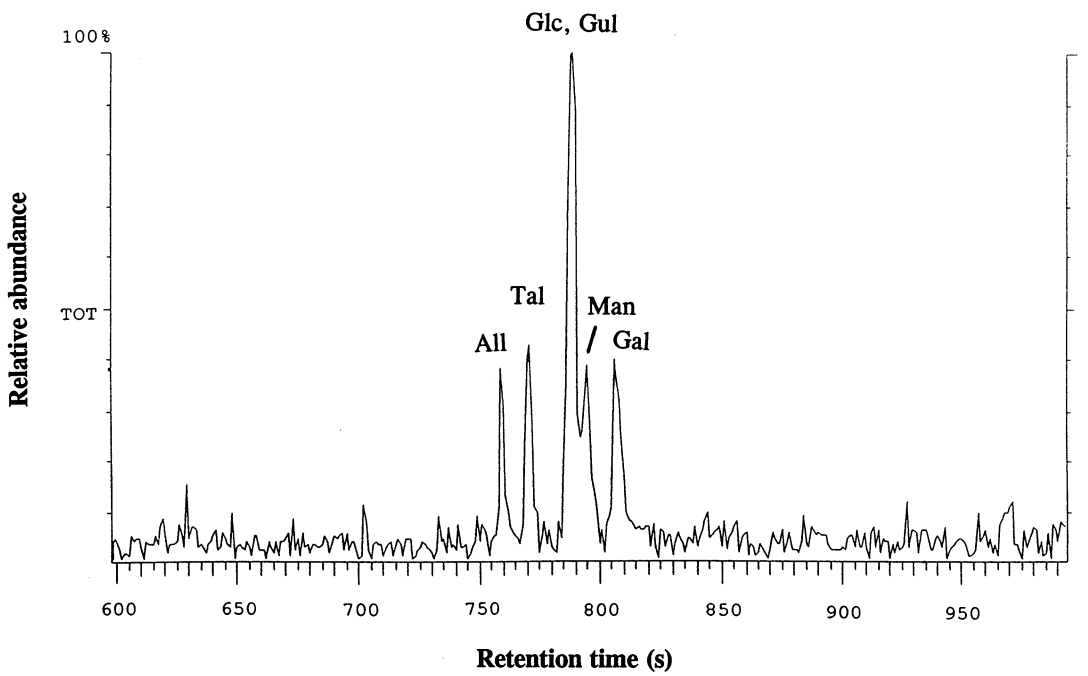


Fig. 4. Sensitivity of GC–MS/MS. Six 1-deoxy-1-hydrazinohexitol heptaacetates (300 fmol each) were chromatographed with detection by CIMS/MS (optimized isobutane pressure, positive ion) in the full scan mode. The m/z 431 ion was selected for fragmentation, with the plot showing the total ion current derived from the precursor ion. Full spectra, with background subtraction applied, were similar to Fig. 3(a). Chromatography was performed on a 30 m DB-5 ms column (0.25 mm i.d., 0.25 μm film thickness), with a constant linear velocity of He of 40 cm/s: injector temperature, 290 °C; oven temperature, 90 °C for 1.5 min during transfer from the liner, then increased at 40 °C/min to an isothermal plateau of 250 °C. Ion-trap conditions were similar to those in Fig. 3.

Table 3
^{1}H NMR data of the 1-deoxy-1-hydrazinohexitol hydrochlorides [a]

Sugar	Chemical shift (δ, ppm)								Coupling constant (Hz)								
	H-1a	H-1b	H-2	H-3	H-4	H-5	H-6a	H-6b	$J_{1a,1b}$	$J_{1a,2}$	$J_{1b,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
D-All	3.377	3.285	4.211	3.858	3.740	3.869	3.790	3.655	−12.9	3.2	9.0	5.0	6.5	6.0	3.2	6.9	−11.9
D-Alt	3.333	3.245	4.248	3.625	3.781	3.919	3.786	3.676	−12.8	9.5	3.3	1.9	8.4	4.9	3.4	7.3	−11.8
D-Gal	3.368	3.269	4.277	3.623	3.672	3.954	3.681 [b]	3.681 [b]	−12.9	9.6	3.3	1.5	9.5	1.4	[b]	[b]	[b]
D-Glc	3.300	3.246	4.120	3.821	3.649	3.759	3.815	3.653	−12.9	3.6	9.0	5.2	2.4	8.2	3.0	6.0	−11.8
D-Gul	3.443	3.219	4.050	3.671	3.81 [c]	3.82 [c]	3.723	3.615	−12.9	3.2	9.2	7.7	1.6	[d]	3.4 [e]	6.4 [e]	−11.8 [e]
D-Ido	3.31 [c]	3.28 [c]	4.133	3.708	3.738	3.831	3.708	3.643	[d]	8.0	4.5	3.5	5.2	3.7	4.4	7.0	−11.7
D-Man	3.487	3.240	4.033	3.808	3.76 [c]	3.74 [c]	3.848	3.668	−12.9	3.2	9.2	8.3	1.0	[d]	2.4	5.4	−11.7
D-Tal	3.339	3.296	4.231	3.850	3.598	3.912	3.657 [f]	3.657 [f]	−12.8	3.7	8.7	4.0	8.6	1.9	[f]	[f]	[f]

[a] For a solution in D_2O at 27 °C, relative to internal DSS at 0.000 ppm. Data was derived from 1-D and 2-D (gCOSY) experiments.
[b] H-6a and H-6b were strongly coupled; $1/2\ (J_{5,6a}+J_{5,6b}) = 6.5$ Hz.
[c] Only assigned to two decimal places due to partial strong coupling of the indicated proton pair.
[d] Not determined due to partial strong coupling.
[e] Additional virtual coupling was observed on H-6a, H-6b.
[f] H-6a and H-6b were strongly coupled; $1/2\ (J_{5,6a}+J_{5,6b}) = 6.4$ Hz.

Table 4
^{1}H NMR data of the 2-deoxy-2-hydrazinohexitol hydrochlorides [a]

Sugar of Origin [b]	Chemical shift (δ, ppm)								Coupling constant (Hz)								
	H-1a	H-1b	H-2	H-3	H-4	H-5	H-6a	H-6b	$J_{1a,1b}$	$J_{1a,2}$	$J_{1b,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
D-Fru (A)	4.012	3.859	3.425	4.148	3.703	3.748	3.824	3.658	−12.2	4.0	7.3	5.5	1.7	8.6	2.8	5.6	−11.8
D-Fru (B)	3.937	3.784	3.431	4.125	3.596	3.754	3.838	3.669	−12.5	3.8	6.5	8.0	1.2	8.9	2.9	5.7	−11.8
D-Psi (A)	4.049	3.879	3.514	4.126	3.759	3.876	3.791	3.670	−12.3	3.8	8.0	3.8	8.0	5.3	3.3	6.9	−12.0
D-Psi (B)	3.907	3.864	3.513	3.987	3.83 [c]	3.81 [c]	3.716	3.598	−12.0	5.0	8.2	3.7 [d]	4.7 [d]	[e]	3.8	6.8	−11.9
D-Sor (A)	3.976	3.844	3.416	4.032	3.778	3.824	3.704	3.628	−12.3	4.0	7.3	5.2	3.8	4.4	4.4	6.6	−11.7
D-Sor (B)	3.925	3.799	3.431	3.953	3.708	3.847	3.722	3.625	−12.4	4.1	6.8	6.8	2.1	5.5	4.1	6.3	−11.9
D-Tag (A)	4.040	3.888	3.557	4.140	3.636	3.915	3.664 [f]	3.664 [f]	−12.3	3.7	8.1	3.5	9.6	1.5	[f]	[f]	[f]
D-Tag (B)	3.925	3.888	3.512	3.896	3.780	3.89 [g]	3.666 [h]	3.666 [h]	−12.0	5.1	7.8	3.0	7.7	2.1	[h]	[h]	[h]

[a] For a solution in D_2O at 27 °C, relative to internal DSS at 0.000 ppm. Data was derived from 1-D and 2-D (gCOSY) experiments.
[b] The stereochemistry at C-2 for each specific derivative was not formally defined. Each ketohexose yielded two reduced 2-deoxy-2-hydrazino products, denoted A and B.
[c] Values assigned to only two decimal places due to partial strong coupling.
[d] Values may need to be interchanged.
[e] Not determined due to partial strong coupling.
[f] H-6a and H-6b were strongly coupled; $1/2\ (J_{5,6a}+J_{5,6b}) = 6.3$ Hz.
[g] Value assigned to only two decimal places due to spectral overlap.
[h] H-6a and H-6b were strongly coupled; $1/2\ (J_{5,6a}+J_{5,6b}) = 6.7$ Hz.

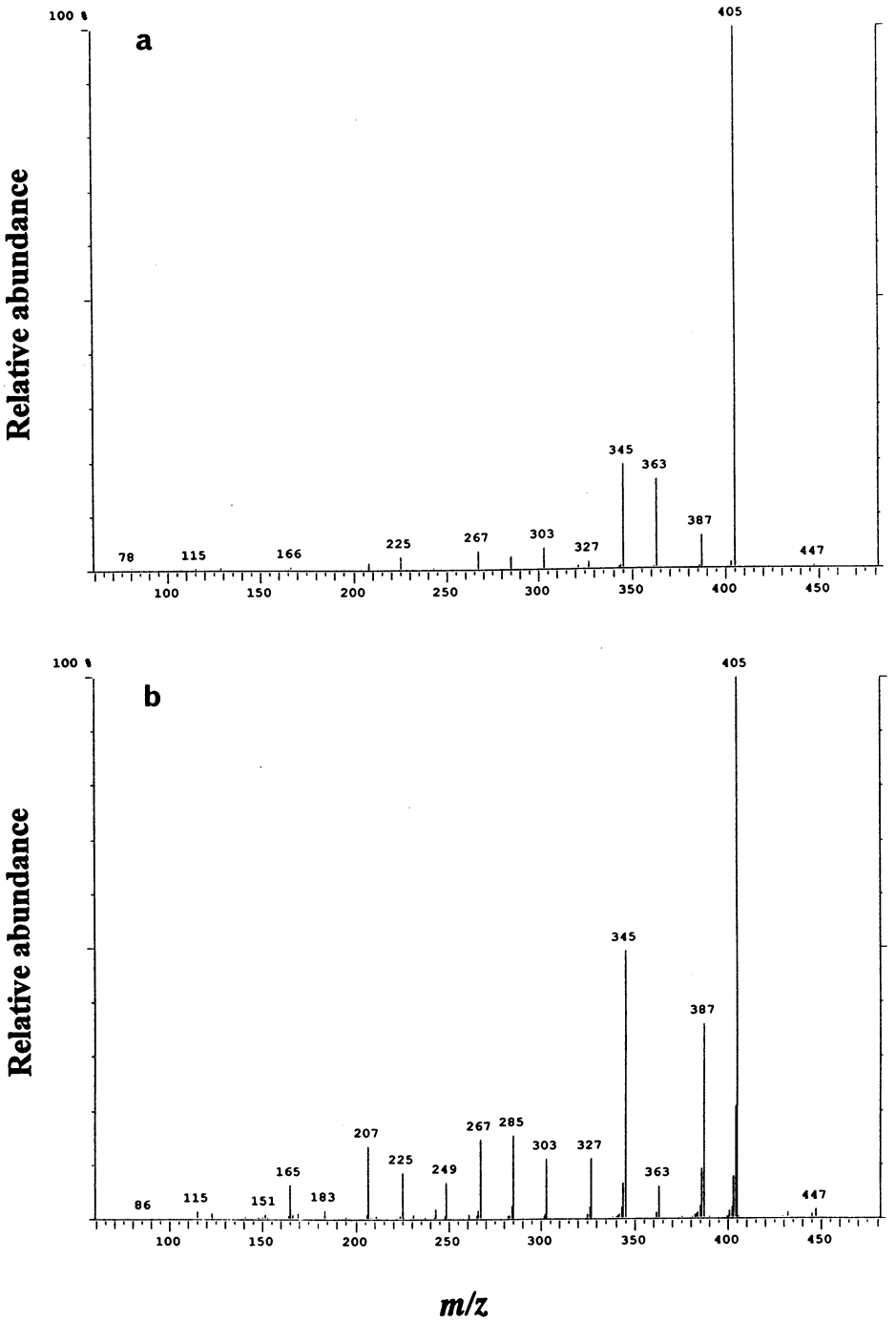


Fig. 5. Chemical ionization-MS/MS in the negative ion mode for 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates, using isobutane as the CI gas, with selection of the major precursor ion m/z 447. (a) The CIMS/MS spectrum for a 1-deoxy-1-hydrazinohexitol heptaacetate. (b) The CIMS/MS spectrum for a 2-deoxy-2-hydrazinohexitol heptaacetate. For each set of derivatives, spectra for all stereochemical variants were similar. Data were collected in the MS/MS mode; source temperature, 225 °C electron energy, 100 eV; trap offset, −7 V; automatic gain control target, 50; ion RF, 47. Precursor ions were selected with an isolation width, 2 amu; isolation time, 8 ms; resonance excitation RF voltage, 0.75 V for 15 ms; q value, 0.225. Product ions were scanned over the ranges shown.

For identification of the reducing monosaccharide at the end of an oligosaccharide, a set of di- and oligosaccharides were examined (Fig. 7). The oligosaccharides were taken through the same set of reactions as for the monosaccharides, with minor changes. Peracetylation of the oligosaccharides now having a 1-deoxy-1-hydrazinohexitol or 2-deoxy-2-hy-

drazinohexitol at the former reducing end was carried out, as the peracetylated products could be easily extracted into chloroform. Hydrolysis of these products cleaved glycosidic linkages and deacetylated the molecules. The 1-deoxy-1-hydrazinohexitols and 2-deoxy-2-hydrazinohexitols were readily isolated from diluted hydrolyzates by binding to, and elution from small Dowex AG5OW (H^+ form) columns. They could be identified unambiguously by NMR (Tables 3 and 4) or, after peracetylation, by GC–MS or GC–MS/MS (Fig. 7). It is important to note that concentration of the hydrolyzates to dryness to remove excess HCl is to be avoided prior to loading on the Dowex AG50W (H^+) column. We observed that concentration, particularly for hydrolyzates of larger oligosaccharides, gives rise to some (about 5–20%) hydrazone products due to a reaction of the hydrazinohexitols with reducing monomers in the concentrated syrup.

Such products were not observed when the 1-deoxy-1-hydrazinohexitols or 2-deoxy-2-hydrazinohexitols were isolated on Dowex AG50W (H^+) directly by dilution of hydrolyzates.

The use of glycerol as a carrier in the concentration step prior to acetylation was important for ensuring solubility of the 1-deoxy-1-hydrazinohexitol or 2-deoxy-2-hy-

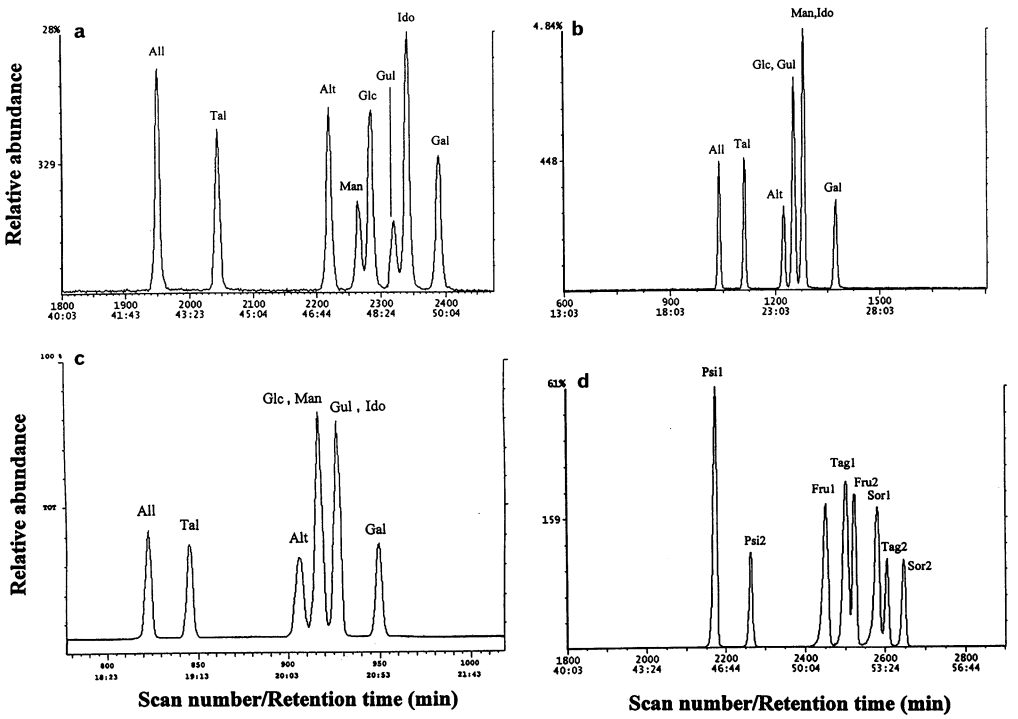


Fig. 6. Gas-chromatographic separations of 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates. Samples ranged from 10 to 50 pmol of individual derivatives. Unless otherwise mentioned, He flow was a constant linear velocity of 40 cm/s; source temperature, 225 °C; column i.d.s, 0.25 mm; film thickness, 0.25 μm; the oven was programmed at 90 °C during transfer from the liner (1.0 min) with an immediate rise after the transfer time at 40 °C/min to an isothermal plateau given for each run below. Injections were splitless, with the split valve opened immediately after the transfer time from the liner. Peaks corresponding to products of specific aldoses or ketoses are indicated. (a) Separation of all eight 1-deoxy-1-hydrazino-D-hexitol heptaacetates on a 105 m DB-1701 column. Detection: CIMS/MS, positive ion mode, reagent gas isobutane, selecting m/z 431 precursor ion, monitoring m/z 329 product ion. GC conditions: injector temperature, 300 °C; transfer time, 1.5 min; isothermal plateau, 280 °C. (b) Chromatography of the eight 1-deoxy-1-hydrazino-D-hexitol heptaacetates on a 20 m DB-17 column (0.18 mm i.d., 0.18 μm film thickness). Detection: EIMS (full scan), m/z 448 intensity plotted. GC conditions: injector temperature, 275 °C; isothermal plateau, 230 °C. (c) Chromatography of the 1-deoxy-1-hydrazino-D-hexitol heptaacetates on a 30 m DB-1301 column. Detection: EIMS (full scan), total ion current plotted. GC conditions: injector temperature, 280 °C; oven programmed at 30 °C/min from 60 °C at $t = 1.0$ min to 200 °C, then at 4 °C/min to 280 °C, then held at 280 °C for a further 10 min. (d) Separation of all eight 2-deoxy-2-hydrazino-D-hexitol heptaacetates on a 100 m DB1 column. Detection: EIMS/MS, selecting the m/z 201 precursor ion, monitoring mass range 70–300 amu, with m/z 159 product ion intensity plotted. GC conditions: injector temperature, 300 °C; He flow, constant linear velocity of 35 cm/s; isothermal plateau, 230 °C.

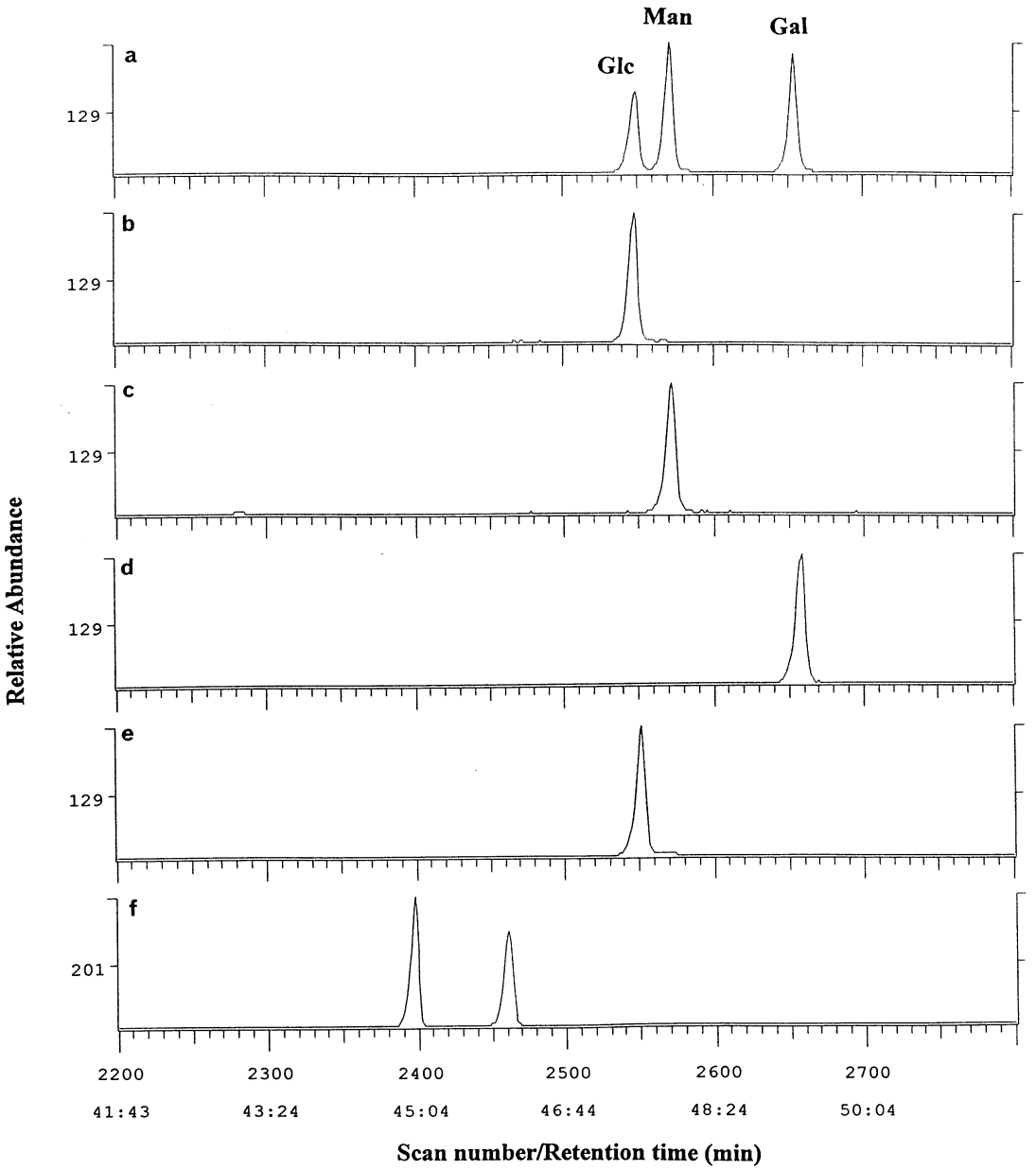


Fig. 7. End-group analysis of some di- and oligosaccharides. The reducing monosaccharide of each di- or oligosaccharide was converted to the 1-deoxy-1-hydrazinohexitol (aldohexoses) or epimeric 2-deoxy-2-hydrazinohexitols (ketohexoses). Oligosaccharide products were peracetylated and, following acid hydrolysis of glycosidic linkages and peracetylation of the reducing-end monomer, samples (20–40 pmol) were examined by GC–MS. Chromatography was performed on a 60 m DB-17 column (0.25 mm i.d., 0.25 μm thickness) at a constant linear velocity of He of 40 cm/s. Injector temperature, 280 °C; transfer time 1.0 min; splitless, split valve opened at 1.0 min; oven initial value, 90 °C, increased at $t = 1.0$ min at 20 °C/min to 220 °C, then at 1 °C/min to 280 °C. Detection; EIMS (full scan), with either m/z 129 (aldose derivatives) or 201 (ketose derivatives) plotted. Iontrap conditions were as in Fig. 1. (a) The three standard 1-deoxy-1-hydrazinohexitol heptaacetates from D-Glc, D-Man, and D-Gal. (b) The reducing-end product from lactose, β-D-Gal-(1→4)-D-Glc. (c) The reducing-end product from mannopentaose, α-D-Man-(1→3)-[α-D-Man-(1→3)-[α-D-Man-(1→6)]-α-D-Man-(1→6)]-D-Man. (d) The reducing-end product from β-D-Gal-(1→6)-D-Gal. (e) The reducing-end product from maltoheptaose $[\alpha\text{-D-Glc-}(1\rightarrow4)]_6$-D-Glc. (f) The reducing-end product from palatinose, α-D-Glc-(1→6)-D-Fru.

drazinohexitol hydrochlorides in the Ac_2O–pyridine solution during acetylation. Removal of these solvents in a Speed-Vac concentrator also removed almost all of the glycerol triacetate, so glycerol appeared to be ideal for this purpose.

3. Conclusions

A method is described for reducing-end group determination of oligosaccharides having either an aldohexose or ketohexose at the reducing end. The need for this method arose from a reaction series we have described, which enables a monosaccharide to be removed from the reducing end of an oligosaccharide [2–4]. With the realization that such a reaction series could be applied repetitively, it was necessary to identify the monomer at the reducing end at any given round or cycle.

The 1-deoxy-1-hydrazinohexitols and 2-deoxy-2-hydrazinohexitols are linear derivatives, unique to each starting aldo- or ketohexose, which satisfied the basic requirements for end-group determination. The derivatives are stable in typical conditions used for hydrolysis of glycosidic linkages. Peracetylation yielded the heptaacetates in 96–98% overall yields, which resulted in unique electron-impact fragmentation patterns for the aldohexose and ketohexose derivatives. When coupled with MS/MS, derivatives from aldoses or ketoses can be independently monitored in the presence of the other. The heptaacetate derivatives can be monitored in subpicomolar quantities by CIMS/MS, although practically, on longer columns, the range of 5–10 pmol was more typical for obtaining full spectra with good signal/noise ratios. The heptaacetate standards were stable in chloroform or acetonitrile at −20 °C for at least 4 years.

All stereoisomers of the D-aldohexose and D-ketohexose derivatives were physically separable by GC, enabling stereochemistries to be assigned in single chromatographic runs. Should enough material be available, the 1-deoxy-1-hydrazinohexitols and 2-deoxy-2-hydrazinohexitols can also be identified as their hydrochlorides by ^{1}H NNR (Tables 3 and 4).

Regarding absolute quantitation, as with most GC–MS methods, the use of isotopically labeled internal standards is to be preferred, due to variability in volumes of single injections. Preparation of suitable standards will be the topic of a future paper.

A point not addressed here is the possibility of identifying enantiomers (D or L forms). Indeed, there are 16 unique D and L 1-deoxy-1-hydrazinohexitols and 16 unique 2-deoxy-2-hydrazinohexitols. We have not, as yet, explored their separations using chiral systems.

4. Experimental

General.—D-Glucose, D-galactose, and D-mannose were from Aldrich. Other D-aldohexoses and D-ketohexoses were from Sigma. Di- and oligosaccharides (Fig. 7) were from Sigma, except the mannopentaose, which was from Dextra laboratories. ^{1}H NMR spectroscopy was performed at 500 MHz on a Varian Inova 500 spectrometer equipped with pulsed field gradients. Spectra of hexose hydrazones, 1-deoxy-1-hydrazinohexitol hydrochlorides, 2-deoxy-2-hydrazinohexitol hydrochlorides, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-hexitols and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)hexitols were accumulated in D_2O (99.96 atom%, Cambridge Isotopes) at 27 °C, with chemical shifts reported relative to a trace (15 μg) of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 0.000 ppm, with an accuracy of ±0.002 ppm. Gradient COSY spectra [12] were recorded with four transients/t_1 increment and a 1.75 s relaxation delay, using echo N-type coherence selection (phase = 1). Gradients were rectangular and applied in the z direction of strength 6 G/cm and 2 ms duration, with gradient rise and fall times of 100 μs. Spectral widths were 3000 Hz in both dimensions, acquiring 4992 points and 512 increments. Spectra of 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates were accumulated in $CDCl_3$ relative to internal Me_4Si.

Electrospray mass spectrometry was performed on a Thermoquest LCQ instrument, either infusing in water, water with 50 μM NaCl, MeCN–water mixtures, or MeCN.

FAB mass spectrometry was performed on a JEOL RX-110 double focusing magnetic sector instrument at 10 kV (located at the University of Washington). High-resolution mass spectra were obtained by peak matching to a standard having a mass of less than 10

amu difference from the unknown; for this spectrometer 99% of values were within ± 7.48 ppm, by mass, of the calculated values (± 2.57σ, where $\sigma = 2.91$ ppm, $n = 187$). Gas chromatography–mass spectrometry (GC–MS) and GC–MS/MS were performed on a Thermoquest GCQ instrument. The GC was equipped with automatic pressure control. Injector liners were modified with an internal sleeve, enabling the end of the GC column to abut to a narrow constriction in the sleeve, which forced carrier gas down the liner. Sleeves were prepared from the narrow end of Pasteur pipettes by pulling under a flame and cutting to fit the liner length. The sleeves, although delicate, increased sensitivity. Septa used were HT X-9 (Alltech), which gave low bleed. Optimized conditions used for the ion trap, chromatographic conditions and column are indicated in the Figure legends. GC columns were from J & W Scientific. Samples were injected in 1 or 2 μL of MeCN or $CHCl_3$.

HPLC was carried out on a Waters 616 pump system with a Waters 486 variable wavelength detector at 200 nm using isocratic elutions at 1.0 mL/min. All Dowex AG50W (H^+ form) refers to X8, 100–200 mesh (Bio-Rad).

Preparation of 1-deoxy-1-hydrazinohexitol heptaacetates and 2-deoxy-2-hydrazinohexitol heptaacetates.—D-Aldoses or ketoses (100 μmol) were dissolved in 2 mL anhyd hydrazine (Pierce). After 16 h under Ar at room temperature (rt), samples were frozen and the hydrazine removed in vacuo on a freeze-dryer (caution: have some ice beforehand in the trap). Products were dissolved in 2 mL water and $NaBH_4$ (200 mg) was added. After 24 h, the sample was placed in an ice-water bath and 4.0 mL of 10% (v/v) AcOH was added dropwise. After 1 h at rt, the sample was transferred quantitatively with water washes to a 500 mL pear-shaped flask (large surface area was important) and concentrated by rotary evaporation to a syrup. The product was concentrated five times to dryness with 20 mL of 5% AcOH in MeOH, followed by three times with 20 mL of MeOH to remove boric acid as trimethyl borate.

To the final dry product was added 100 mL of Ac_2O, followed by heating for 2 h to 100 °C. Although in great excess, this quantity was needed for adequate dissolution of the sodium acetate. Acetic anhydride was removed by rotary evaporation, the product was taken up in 100 mL of $CHCl_3$, and NaOAc was removed with 3 × 50 mL extractions with water. The product was concentrated to dryness, and dissolved in 10 mL of MeCN. Compounds prepared were: 1-deoxy-1-hydrazino-D-allitol heptaacetate, 1-deoxy-1-hydrazino-D-altritol heptaacetate, 1-deoxy-1-hydrazino-D-galactitol heptaacetate, 1-deoxy-1-hydrazino-D-glucitol heptaacetate, 1-deoxy-1-hydrazino-D-gulitol heptaacetate, 1-deoxy-1-hydrazino-D-iditol heptaacetate, 1-deoxy-1-hydrazino-D-mannitol heptaacetate, and 1-deoxy-1-hydrazino-D-talitol heptaacetate. The 2-deoxy-2-hydrazinohexitol heptaacetates were prepared as the 2-epimeric pairs; from D-fructose, 2-deoxy-2-hydrazino-D-glucitol heptaacetate and 2-deoxy-2-hydrazino-D-mannitol heptaacetate; from D-tagatose, 2-deoxy-2-hydrazino-D-galactitol heptaacetate and 2-deoxy-2-hydrazino-D-talitol heptaacetate; from D-sorbose, 2-deoxy-2-hydrazino-D-gulitol heptaacetate and 2-deoxy-2-hydrazino-D-iditol heptaacetate; from D-psicose, 2-deoxy-2-hydrazino-D-allitol heptaacetate and 2-deoxy-2-hydrazino-D-altritol heptaacetate. Isolated yields of products were from 96–103%, with the proviso that the 2-deoxy-2-hydrazinohexitol heptaacetate yields represented that of the mixtures of 2-epimers. Compounds at this stage were analyzed by GC–MS and GC–MS/MS, after 100–10,000-fold dilution (see Section 2). They contained 2–4% of the respective alditol acetates and residual *N,N′*-diacetylhydrazine. ^1H NMR spectra ($CDCl_3$) were complicated and, in some cases, broadened due to cis–trans conformers about the hydrazino nitrogen–carbonyl carbon bonds. High-resolution NMR spectra were obtained after deacetylation (below).

*Preparation of 1-deoxy-1-(*N,N′*-diacetylhydrazino)hexitols and 2-deoxy-2-(*N,N′*-diacetylhydrazino)hexitols.*—D-Hexoses (either aldoses or ketoses) were treated with hydrazine and reduced with borohydride as already described for the heptaacetates. Samples were cooled on an ice-water bath and glacial

AcOH (0.4 mL) was added dropwise. The sample was warmed to rt, diluted with 2 mL of water and after 1 h $NaHCO_3$ (0.4 g) was added and dissolved. Acetic anhydride (0.2 mL) was added with gentle swirling to dissolve it, and after 10 min another 0.2 mL was added. After 1 h, the sample was diluted with 10 mL of water, and loaded on a 25-mL column of Dowex AG50W (H^+ form), followed by 5×20 mL washes with water to remove Na^+. The eluate was concentrated to dryness, and boric acid was removed as trimethylborate as already described. To obtain pure title products, samples were chromatographed by HPLC on a column of GlycoPak N (Waters, 7.8×300 mm), eluting isocratically with 17:3 MeCN–water. 1-Deoxy-1-(*N*,*N*′-diacetylhydrazino)hexitols chromatographed as single peaks eluting between 41 and 46 min.

Yields were essentially quantitative. Compounds prepared were: 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-allitol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-altritol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-galactitol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-glucitol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-gulitol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-iditol, 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-mannitol, and 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-talitol.

Positive ion FABMS (3-nitrobenzyl alcohol, matrix) of 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-hexitols: *m*/*z* 281 $(M + H)^+$, and, in the presence of NaOAc, 303 $(M + Na)^+$. Linked-scan positive B/E (10 kV) of $(M + H)^+$ yielded product ions of *m*/*z* 263 $(M + H–H_2O)^+$, 239 $(M + H–ketene)^+$, and 221 $(M + H–ketene–H_2O)^+$. For both the gluco and galacto configurations, HRFABMS gave $(M + H)^+$ *m*/*z* 281.135 (Calcd 281.135), and for the gluco configuration $(M + Na)^+$, *m*/*z* 303.117 (Calcd 303.117). ^1H NMR (D_2O) resulted in very broad signals. Compounds were deacetylated to give high resolution NMR spectra (below). The 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-hexitols chromatographed (HPLC) as two peaks representing the respective 2-epimers between 31 and 36 min, except for sorbose, which gave a broad peak at 34 min containing both 2-epimers. Overall yields of 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-hexitols were only 10–15%. An additional HPLC peak was observed, for all products, at 19.5 min (including products generated from the aldoses, above), which was *N*,*N*′-diacetylhydrazine. Compounds prepared: from D-fructose, 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-glucitol and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-mannitol; from D-tagatose, 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-galactitol and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-talitol; from D-sorbose, 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-gulitol and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-iditol; from D-psicose, 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-allitol and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-altritol. Positive ion FABMS (3-nitrobenzylalcohol, matrix) of 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-hexitols: *m*/*z* 281 $(M + H)^+$, and, with NaOAc, 303 $(M + Na)^+$. ^1H NMR (D_2O) gave broad signals; compounds were deacetylated to yield high resolution spectra (below). The HPLC-pure products of the 1-deoxy-1-(*N*,*N*′-diacetylhydrazino)-D-hexitols and 2-deoxy-2-(*N*,*N*′-diacetylhydrazino)-D-hexitols were O-acetylated as follows. To 1.0 μmol of each was added 100 μL of dry pyridine (Pierce, silylation grade) and 50 μL of Ac_2O. Samples were kept under Ar for 24 h, then solvents were removed on a Speed-Vac rotary concentrator.

Starting compounds, in each case, were converted quantitatively into the 1-deoxy-1-hydrazinohexitol heptaacetates or 2-deoxy-2-hydrazinohexitol heptacetates (as above). They were taken up in $CHCl_3$ or MeCN (1.0 mL) and serially diluted for GC–MS and GC–MS/MS analyses.

Preparation of 1-deoxy-1-hydrazinohexitol hydrochlorides and 2-deoxy-2-hydrazinohexitol hydrochlorides.—Either the heptaacetate derivatives or the *N*,*N*′-diacetyl derivatives described above (5 μmol) were treated under Ar with 1 mL 2.0 M HCl for 3.5 h at 100 °C. Samples were diluted with 5 mL of water and rotary evaporated to dryness. They were transferred quantitatively with 4×1 mL washes of 10 mM HCl to a 1-mL column of Dowex AG50W (H^+ form, prepared beforehand in Pasteur pipettes with glass wool plugs by washing with 10 column volumes of 4.0 M HCl, then 20 column volumes of water). After

washing with another 5 mL of 10 mM HCl, the products were eluted with 8 mL of 4.0 M HCl. The final eluate was concentrated by rotary evaporation and examined by ^{1}H NMR spectroscopy in D_2O relative to DSS, including standard 1-D and 2-D gradient COSY spectra (Tables 3 and 4). Electrospray MS, for all compounds, gave m/z 197, $(M+H)^+$.

Identification of a hexose at the reducing end of oligosaccharides.—Di- and oligosaccharides were derivatized with hydrazine, reduced, and peracetylated as described for the preparation of the 1-deoxy-1-hydrazinohexitol heptaacetates or 2-deoxy-2-hydrazinohexitol heptaacetates, with the differences being that treatment with Ac_2O was for 3 h at 100 °C, rather than 2 h, and that quantities of oligosaccharides used were the same mass, rather than molarity, as the hexoses. Peracetylation was carried out solely to enable rapid separation of product from NaOAc by partitioning into $CHCl_3$. Peracetylated products were examined by electrospray MS in the presence of NaCl, which gave appropriate ions for all peracetylated oligosaccharide end-group derivatized products: disaccharide derivatives, m/z 801.2 (Calcd 801.2, $(M+Na)^+$); mannopentaose derivative, m/z 1665.4 (Calcd 1665.5 $(M+Na)^+$); maltoheptaose derivative, m/z 1132.6 (Calcd 1132.3, $(M+2Na)^{2+}$). To a peracetylated, hydrazine-end labeled oligosaccharide (the mass equivalent of 1 μmol of a 1-deoxy-1-hydrazinohexitol heptaacetate) was added 250 μL of 2.0 M HCl, and the sample heated under Ar for 3.5 h at 100 °C, with occasional shaking. The sample was cooled, diluted to 20 mL with water, and passed over a 1.0-mL Dowex AG50W (H^+ form) column (as already described for 1-deoxy-1-hydrazinohexitol hydrochlorides), followed by a wash with 5 mL of 10 mM HCl, then an elution with 8 mL of 4.0 M HCl. After rotary evaporation, the hydrochloride product was examined by ^{1}H NMR in D_2O. Small portions (5–50 nmol) of end-group products (either 1-deoxy-1-hydrazino- or 2-deoxy-2-hydrazinohexitol hydrochlorides) in 5–50 μL of water were mixed with 10 μL of a 5% (v/v) aqueous solution of glycerol. Samples were concentrated to a syrup on a Speed-Vac concentrator. Acetic anhydride (50 μL) was added, followed by pyridine (100 μL, Aldrich product 27,040–7). The sample was capped under Ar. After 24 h with occasional mixing, the solvent was removed on the concentrator, and the sample taken up in 100 μL to 1 mL of MeCN for GC–MS or GC–MS/MS injection. Depending on the GC column and quantity of product, further dilution was sometimes necessary.

Acknowledgements

Research was supported by the University of Colorado, the University of Colorado Cancer Center, and Takara Shuzo Co., Ltd. An NCI equipment supplement to the Colorado Cancer Center core grant NIH-P30-CA46934-10, and the Howard Hughes Medical Institute provided funds for high-field NMR spectrometers used for these studies. The authors thank Dr S.B. Levery, M.E. Salyan, and W. Loyd for their contributions in preliminary stages of this work.

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